Supporting Information

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SI Text

β-Galactosidase Assay. Embryos or tissues were fixed in fresh 0.2% glutaraldehyde and stained with a solution containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 1 mg/ml X-gal substrate at 37°C overnight. Postfixation was performed in 4% paraformaldehyde (PFA) at 4°C overnight. For histological analysis, tissue was dehydrated, cleared (Histo-Clear; National Diagnostics), embedded in paraffin, sectioned, and counterstained with nuclear fast red (Vector Laboratories).

Immunofluorescence Staining. Tissues were fixed overnight in freshly prepared 4% PFA, rinsed in PBS, saturated with 20% sucrose for cryoprotection, embedded in agar, and frozen in OCT. Twelve-micrometer cryosections were fixed in methanol at room temperature for 5 min, permeabilized with 0.05% Tween-20 (in PBS) for 2 min, rinsed in PBS, then blocked in 2% normal goat serum (NGS) or normal donkey serum for 2–8 h. Sections were incubated with primary antibodies at 4°C overnight, rinsed twice in PBS + 0.1% BSA, then incubated with secondary antibodies for 2–8 h at room temperature, protected from light. Nuclei were counterstained with 10 μg/ml Hoechst 33258 in H_2O and slides were mounted in Mowiol with 2.5% DABCO.

For cells in culture, cells were fixed by incubating in freshly prepared 4% PFA for 15 min at room temperature, then permeabilized with 1:1 methanol/acetone for 1 min. Cells were blocked in 2% NGS for 1–4 h at 4°C, then incubated with primary antibodies at 4°C overnight. After two rinses in PBS + 0.1% BSA, cells were incubated with secondary antibodies 1–4 h at room temperature protected from light. Nuclei were counterstained with 10 μg/ml Hoescht 33258 in H_20. For BrdU dual-labeling experiments, cells were first incubated with other primary and secondary antibodies, then postfixed in 4% PFA for 15 min at room temperature. To access BrdU antigen, DNA was denatured in 2 M HCl for 5 min at room temperature. Cells were rinsed twice with PBS to neutralize residual acid, and then stained according to standard protocol.

Primary antibodies used for these studies included rabbit anti-β-galactosidase (1:500; MP Biomedical 55976), goat anti-Shh (1:100; R&D SystemsAF445), rabbit anti-Dhh (1:100; Santa Cruz Biotechnology sc13089), rabbit anti-SM-MHC (1:100; Biomedical Technologies BT-562), rabbit anti-SRF (1:100; Santa Cruz Biotechnology sc335), rabbit anti-GKLF/Klf4 (1:50; Santa Cruz Biotechnology sc20691), mouse anti-SMαActin (1:100; Sigma A2547), rat anti-Sca1 (1:100; BD Pharmingen 553333), rabbit anti-Ihh (1:100; Santa Cruz Biotechnology sc13088), and rat anti-PECAM-1 (1:100; BD Pharmingen 550274). All secondary antibodies were AlexaFluor-conjugated (Invitrogen) and used at a dilution of 1:400 in PBS. Immunofluorescence staining was visualized with a Leica BM IRB inverted epifluorescence microscope, with images captured by a QImaging Retiga 1300 digital camera. Confocal images were obtained with a Zeiss LSM5 Pascal laser scanning confocal microscope and processed with Image J and Adobe Photoshop.

Cell Proliferation and Differentiation Assays. For experiments to determine whether AdvSca1 cells can express SMC differentiation markers in the absence of cell proliferation, AdvSca1 cells were isolated by immunoselection with magnetic MicroBeads and a MACS cell separation system (Miltenyi Biotech) and cultured for 5 days in DMEM + 10% bovine calf serum + 1× antibiotic/antimycotic (Sigma) to allow cells to attach and spread on the culture substrate. At 5 days, cell proliferation was arrested by the addition of 50 μM aphidicolin (in DMSO; Sigma A0781) or 8 mM hydroxyurea (in H_2O; Sigma, H8627). To verify growth arrest, 25 μM BrdU (BD Pharmingen 550891) was added to each well with or without inhibitors.

Histological Staining. Alizarin red S and von Kossa staining procedures were similar to those described (1). For alizarin red staining, cultures were washed three times in 150 mM NaCl, then fixed in ice-cold 70% ethanol for 1 h. Cells were rinsed with ddH_20, incubated with alizarin red staining solution (2% alizarin red in H_2O, pH 4.3) at room temperature for 10 min, then washed five times in ddH_2O and photographed. For von Kossa staining, cultures were rinsed twice in ddH_2O, then incubated in 1% (wt/vol) sodium thiosulfate to remove unreacted silver. After a rinse in ddH_20, cells were counterstained in van Gieson solution (0.6% picric acid, 0.0375% acid fuchsin in H_20) for 5 min then washed in 70% ethanol, dried, and photographed.

RT-PCR Analysis. The sequences for primers used for RT-PCR analysis of AdvSca1 gene expression are provided in Table S1. Unless otherwise indicated, primer sequences were designed for this study based on mouse genomic sequence available through Ensembl (release 45, June 2007; ref. 2). Primers were designed with the aid of Primer3 software (v. 0.4.0) (3).

AdvSca1 Cell Count from *Shh*$^{-/-}$ Embryos. Embryos were isolated at E18.5. The aorta was isolated and divided by anatomical region into ascending and descending portions (see schematic in Fig. 5*E*). *Shh*$^{-/-}$ embryos were pooled and matched to a control group comprised of an equal number of WT littermates. Tissue was digested with 14 mg/ml collagenase type 2 (Worthington) and 0.75 mg/ml elastase (Roche) in HBSS for 2 h at 37°C with gentle rocking. The cell suspension was filtered (70 μm), and cells in the filtrate were pelleted at 300 × *g*, rinsed in PBS + 0.5% BSA, and counted. Sca1$^+$ cells were isolated by using anti-Sca1 immunomagnetic MicroBeads and a MACS cell separation system (Miltenyi). The number of isolated Sca1$^+$ cells was determined and compared with initial cell counts to calculate the fraction of Sca1$^+$ cells.

1. Akune T, Ohba S, Kamekura S, Yamaguchi M, Chung U, Kubota N, Terauchi Y, Harada Y, Azuma Y, Nakamura K, et al (2004) PPARgamma insufficiency enhances osteogenesis through osteoblast formation from bone marrow progenitors. *J Clin Invest* 113:846–855.
2. Hubbard T, Aken B, Beal K, Ballester B, Caccamo M, Chen Y, Clarke L, Coates G, Cunningham R, Cutts T (2007) Ensembl 2007. *Nucleic Acids Res* 35:D610–D617.
3. Rozen S, Skaletsky H (2000) Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* 132:365–386.

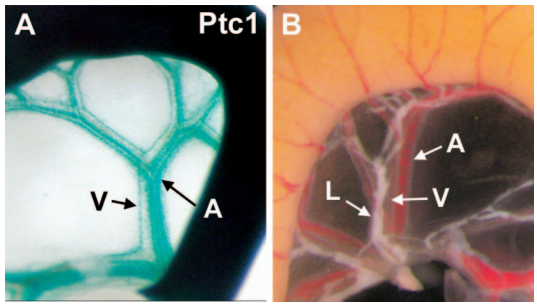

Fig. S1. *Ptc1*^{lacZ} activity is absent in lymphatic vessels. (*A*) Whole mount-stained images for β-galactosidase activity in a mesenteric spread from a *Ptc1*^{lacZ} mouse at P2. The arteries are strongly positive, the veins are weakly positive, and the lymphatic vessels (shown in *B*) are negative. (*B*) A mesenteric spread after dissection from a *Ptc1*^{lacZ} mouse at P2 taken at an equivalent level as shown in *A*. The lymphatic vessels are identified by milky white contents after nursing. These vessels are negative for *Ptc1*^{lacZ} activity. (Magnification: ×7.)

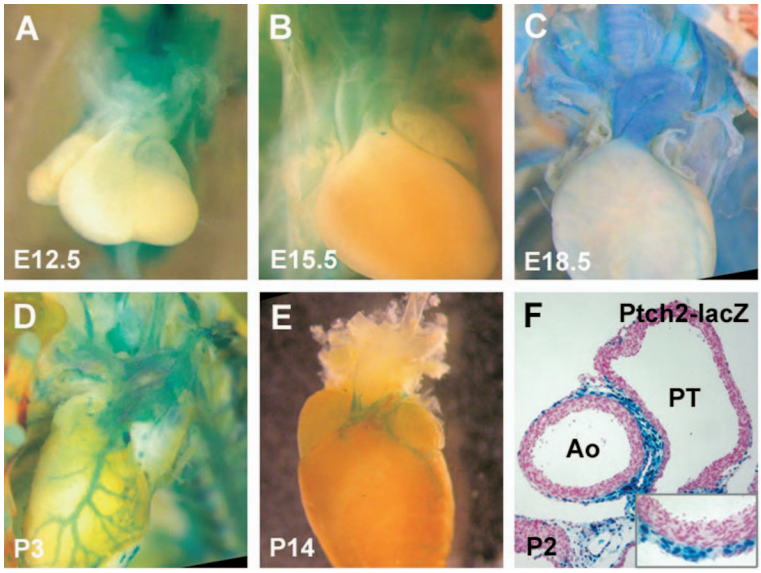

Fig. S2. Developmental time course of *Ptc*^{lacZ} expression in the aortic root region. (*A–E*) Whole-mount hearts and vessels from *Ptc1*^{lacZ} mice were isolated and stained for β-galactosidase activity on embryonic days 12.5 (*A*), 15.5 (*B*), 18.5 (*C*), and postnatal days 3 (*D*) and 14 (*E*). The ventral surface of the heart is shown in *A–C* and *E*, while the heart in *D* has been rotated slightly so image shows the left coronary artery. In some images, atria have been removed to better view the outflow tract vessels. (*F*) A cross-section of the aortic root from a postnatal day 2 *Ptc2*^{lacZ} heart reveals β-galactosidase-positive cells localized to the adventitial layer of the artery wall (compare with *Ptc1*^{lacZ} staining shown in Fig. 1*C*). (*Inset*) *Ptc2*^{lacZ} activity in the descending thoracic aorta (at a similar level as shown for *Ptc1*^{lacZ} in Fig. 1*C*). Counterstain is nuclear fast red. Ao, aorta; Pt, pulmonary trunk. (Magnification: *A*, ×10; *B–D*, ×6; *E*, ×4; *F* ×10; *Inset*, ×40.)

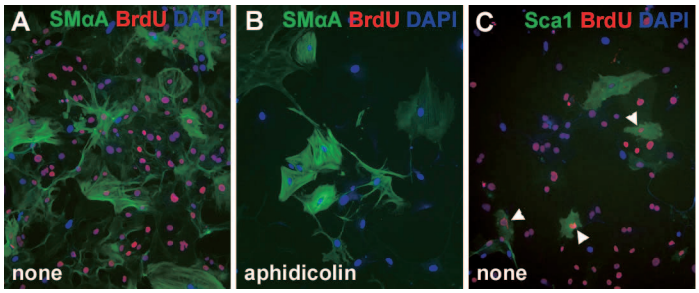

Fig. S3. AdvSca1 cell differentiation in the absence of cell division. (*A*) Isolated AdvSca1 cells were cultured in serum-containing medium in the presence of 10 μM BrdU. Proliferating cells are identified by red nuclei. (*B*) The addition of 50 μM aphidicolin strongly inhibited cell proliferation, as indicated by the absence of BrdU staining (red). The up-regulation of SMαActin (green) in AdvSca1 cells does not require cell proliferation. (*C*) AdvSca1 cells can also undergo self-renewal *in vitro*, as indicated by BrdU and ScaI costaining (white arrowheads). (Magnification: $\times$10.)

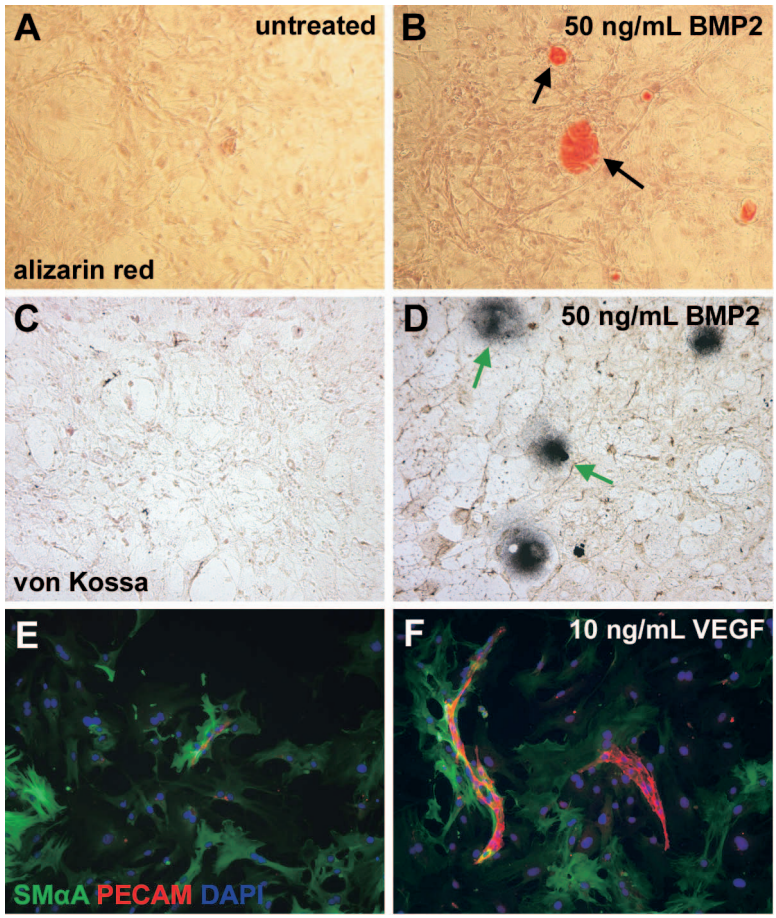

Fig. S4. Endothelial and osteogenic potentials of AdvSca1 cells *in vitro*. Isolated AdvSca1 cells were cultured in medium without (*A*, *C*, and *E*) or with the addition of BMP2 (50 ng/ml, 20 days) (*B* and *D*) or VEGF-A (10 ng/ml, 9 days) (*F*). (*A* and *B*) Alizarin red forms a red-orange complex with calcium ions indicating the formation of calcified nodules (black arrows in *B*). (*C* and *D*) Von Kossa staining indicates the presence of mineralized colonies by the formation of silver salts, which are reduced to black metallic silver (green arrows in *D*). (*E* and *F*) Immunostaining for PECAM1 (red) and SMαActin (green) in AdvSca1 cell cultures. PECAM1-positive endothelial cells are more abundant and have a greater complexity of organization in AdvSca1 cultures treated with VEGF-A (*F*) than in nontreated cultures (*E*). The overall frequencies of alizarin red- or PECAM1-positive cells per AdvSca1 isolation were at least 10-fold lower than that observed for SMαActin-positive cells. (Magnification: $\times$10.)

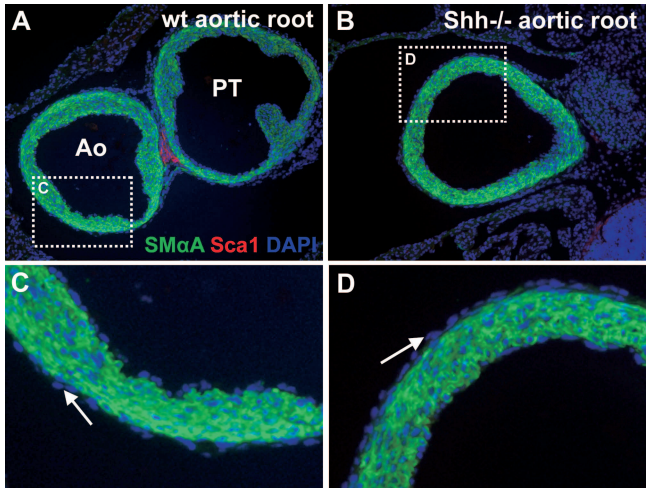

Fig. S5. Development of aortic adventitia in $Shh^{-/-}$ mice. (*A* and *C*) Aortic root images from WT mice at E18.5. The boxed segment in *A* is shown at higher magnification in *C*. The adventitia is identified as DAPI-positive nuclei (blue) outside the SMαActin-positive medial layer (green). Although the adventitial layer is still forming at E18.5, a similar number of DAPI-positive nuclei are found in the adventitia surrounding the aortic root in both WT (*A* and *C*) and $Shh^{-/-}$ mice (*B* and *D*). Shh-deficient embryos do not form a septation complex in the truncus arteriosus (1). We cannot at this time distinguish between direct effects caused by the loss of Shh signaling in AdvSca1 cells themselves versus indirect effects of Shh deficiency on the developing outflow arteries as the cause of the loss of AdvSca1 cells from the adventitia surrounding the vessels shown in Fig. 5. (Magnification: *A* and *B*, $\times$10; *C* and *D*, $\times$30.)

1. Washington Smoak I, *et al.* (2005) Sonic hedgehog is required for cardiac outflow tract and neural crest cell development. *Dev Biol* 283:357–372.

Table S1. RT-PCR primer sequences

Gene	Forward primer	Reverse primer	Product size, bp	Ref.
β-actin	TGTTACCAACTGGGACGACA	CTCTCAGCTGTGGTGGTGAA	393	1
Boc	cccagaagctccagacagac	tgtccccttctaccatgtcc	500	
Calponin	CACCAACAAGTTTGCCAG	TGTGTCGCAGTGTTCCAT	213	2
CD140b (PDGFR-β)	AGCTACATGGCCCCTTATGA	GGATCCCAAAAGACCAGACA	367	
CD34	ttgacttctgcaaccacgga	tagatggcaggctggacttc	300	3
Cdo	aggggagagtgagttcagca	cctggtcagggagtttgtgt	493	
Csrp1	AGTCTCTGGGCATCAAGCAT	CCGCTGATGAAAAGCTTAGG	376	
Csrp2	GCATGGTTTGCAGGAAAAAT	CCCACACCTGGAACACTTCT	260	
Dhh	ATGCCCAATTGACAGGAGAG	GGCCTTCGTAGTGGAGTGAA	500	
Disp1	CAAGAGGGACCACGATAGGA	GTGCCGTTTTGGTAGTGCTT	505	
Flk1	tctgtggttctgcgtggaga	gtatcatttccaaccaccct	270	3
Foxo4	TGTAACAGGTCCTCGGAAGG	GACAGACGGCTTCTTCTTGG	394	
Gli1	TGGAAGGGGACATGTCTAGC	ATGGCTTCTCATTGGAGTGG	501	
Gli2	CCTCCAACCTCAACAAGAGC	CTGCAGGAGGGAGAAAACTG	403	
Gli3	CCGTTCAAAGCCCAGTACAT	TCTTCACCTGGAGGCACTCT	505	
Hhip	CCGTGGATCGACATCCTACT	GGGCAGGTTGAACTGTGACT	498	
Ihh	GGCCATCACTCAGAGGAGTC	ATATTGGCCTGGTTGCACAT	501	
Kit	ggctcataaatggcatgctc	cttccattgtacttcatacatg	400	3
Klf4	ATTAATGAGGCAGCCACCTG	GGAAGACGAGGATGAAGCTG	400	
MRTF-A	CCAGGCTGGCTGATGACCTCAATG	CTGTGATTTCTCGCTGGCAGACTTG	473	2
MRTF-B	ACCCCAGCAGTTTGTTGTTC	TGGAATGACTCAGCAAGTCG	488	
Msx1	GCTGGAGAAGCTGAAGATGG	AGGGGTCAGATGAGGAAGGT	391	
Msx2	AGACATATGAGCCCCACCAC	GGGAGCACAGGTCTATGGAA	365	
Myocardin	ATGCACCAAACACACCTCAA	GCTGCCAAAGTGGTAGAAGC	384	
Ptch1	CATTGGCAGGAGGAGTTGAT	CCTGAGTTGTCGCAGCATTA	499	
Ptch2	TCCCCCAGAGCTCTTCTACA	GGGATGGCACTCAGTTTGAT	501	
Sca1	CTCGAGGATGGACACTTCT	GGTCTGCAGGAGGACTGAGC	400	
Shh	TCTGTGATGAACCAGTGGCC	GCCACGGAGTTCTCTGCTTT	241	4
SM α-actin	ACGGCCGCCTCCTCTTCCTC	GCCCAGCTTCGTCGTATTCC	415	
SM-MHC	GACAACTCCTCTCGCTTTGG	GCTCTCCAAAAGCAGGTCAC	201	5
SM22α	tccagtccacaaacgaccaagc	gaattgagccacctgttccatctg	328	6
Smo	GGCTGGAGTAGTCTGGTTCG	TGGCTTGGCATAGCACATAG	500	
SRF	CTACCAGGTGTCGGAATCTGA	CCAGACGGTGCTGTCAGGAACA	651	2
Sufu	CAAAGGCATTGAGACAGACG	GCATACGGGTGTTCCTCAGT	507	

1. Kume T, Jiang H, Topczewska JM, Hogan B (2001) The murine winged helix transcription factors, Foxc1 and Foxc2, are both required for cardiovascular development and somitogenesis. *Genes Dev* 15:2470–2482.
2. Brunelli S, *et al.* (2004) Msx2 and necdin combined activities are required for smooth muslce differentiation in mesoangioblast stem cells. *Circ Res* 94:1571–1578.
3. Minasi MG, *et al.* (2002) The mesoangioblast: a multipotent, self-renewing cell that originates from the dorsal aorta and differentiates into most mesodermal tissues. *Development* 129:2773–2783.
4. Krebs O, *et al.* (2003) Replicated anterior zeugopod (raz): A polydactylous mouse mutant with lowered Shh signaling in the limb bud. *Development* 130:6037–6047.
5. Hu Y, *et al.* (2004) Abundant progenitor cells in the adventitia contribute to atherosclerosis of vein grafts in ApoE-deficient mice. *J Clin Invest* 113:1258–1265.
6. Sinha S, Hoofnagle M, Kingston P, McCanna M, Owens G (2004) Transforming growth factor-β 1 signaling contributes to development of smooth muscle cells from embryonic stem cells. *Am J Physiol* 287:C1560–C1568.